



In Vitro Assays for Anti-Toxin Therapies

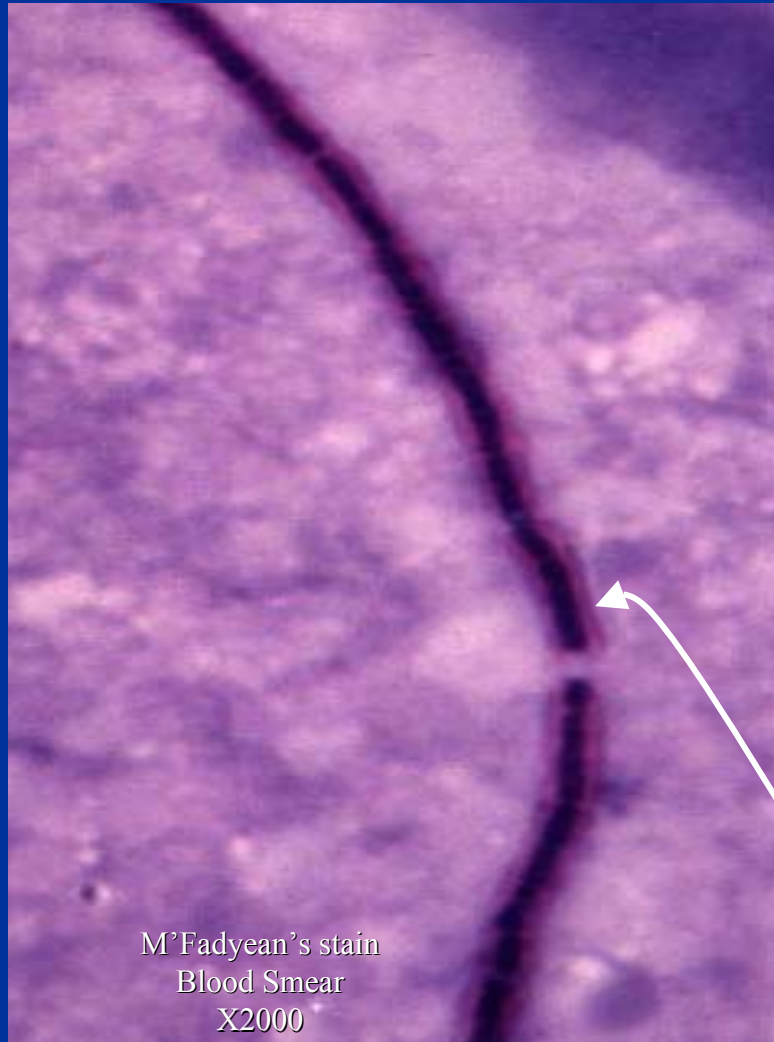
June 2004

Conrad P. Quinn

CDC

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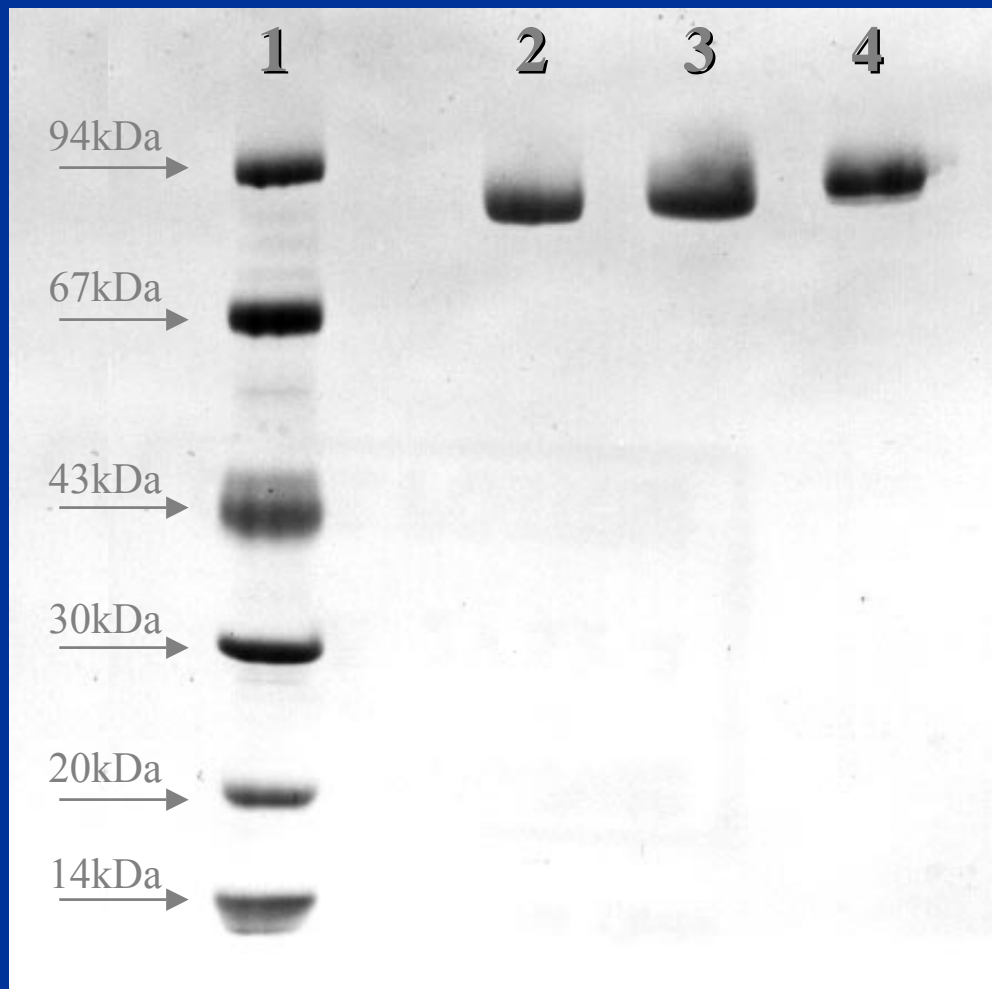
Bacillus anthracis



M'Fadyean's stain
Blood Smear
X2000

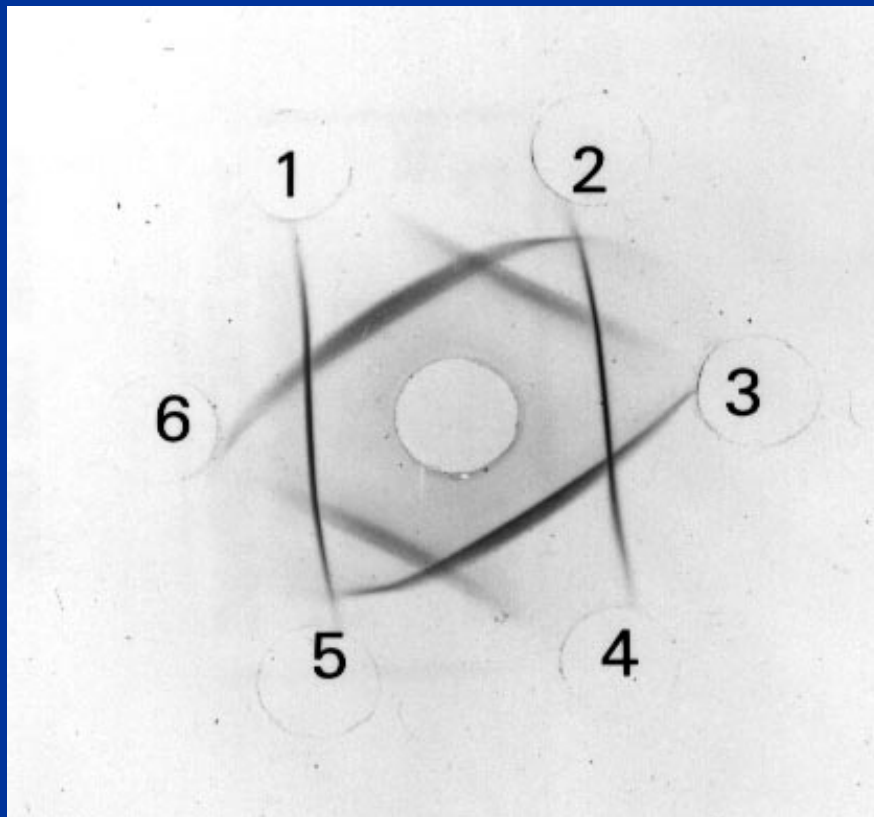
- Gram positive, spore former
- 0.8-1.5 μ m X 8-10 μ m
- Non-motile
- Penicillin sensitive
- Gamma-phage sensitive
- Non-haemolytic
- Tri-partite protein toxin
- γ -linked, poly-D-glutamic acid capsule

SDS-PAGE Analysis of Purified Native Anthrax Toxin Components



1. Molecular wt. Standards
2. PA - 83 kDa
3. LF - 90 kDa
4. EF - 89 kDa

Ouchterlony DID of Purified Anthrax Toxin Components



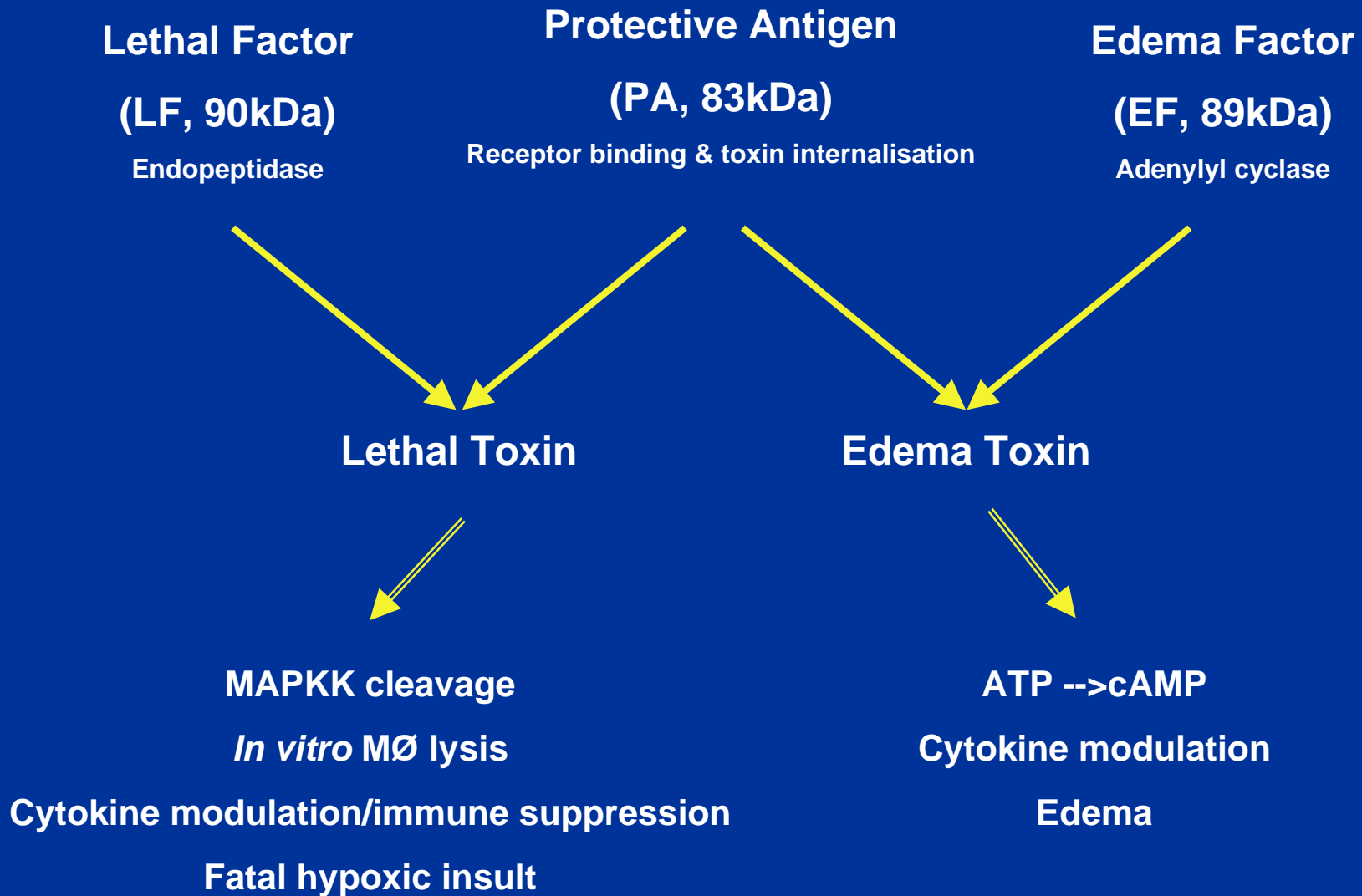
1 & 4. = PA

2 & 5. = EF

3 & 6. = LF

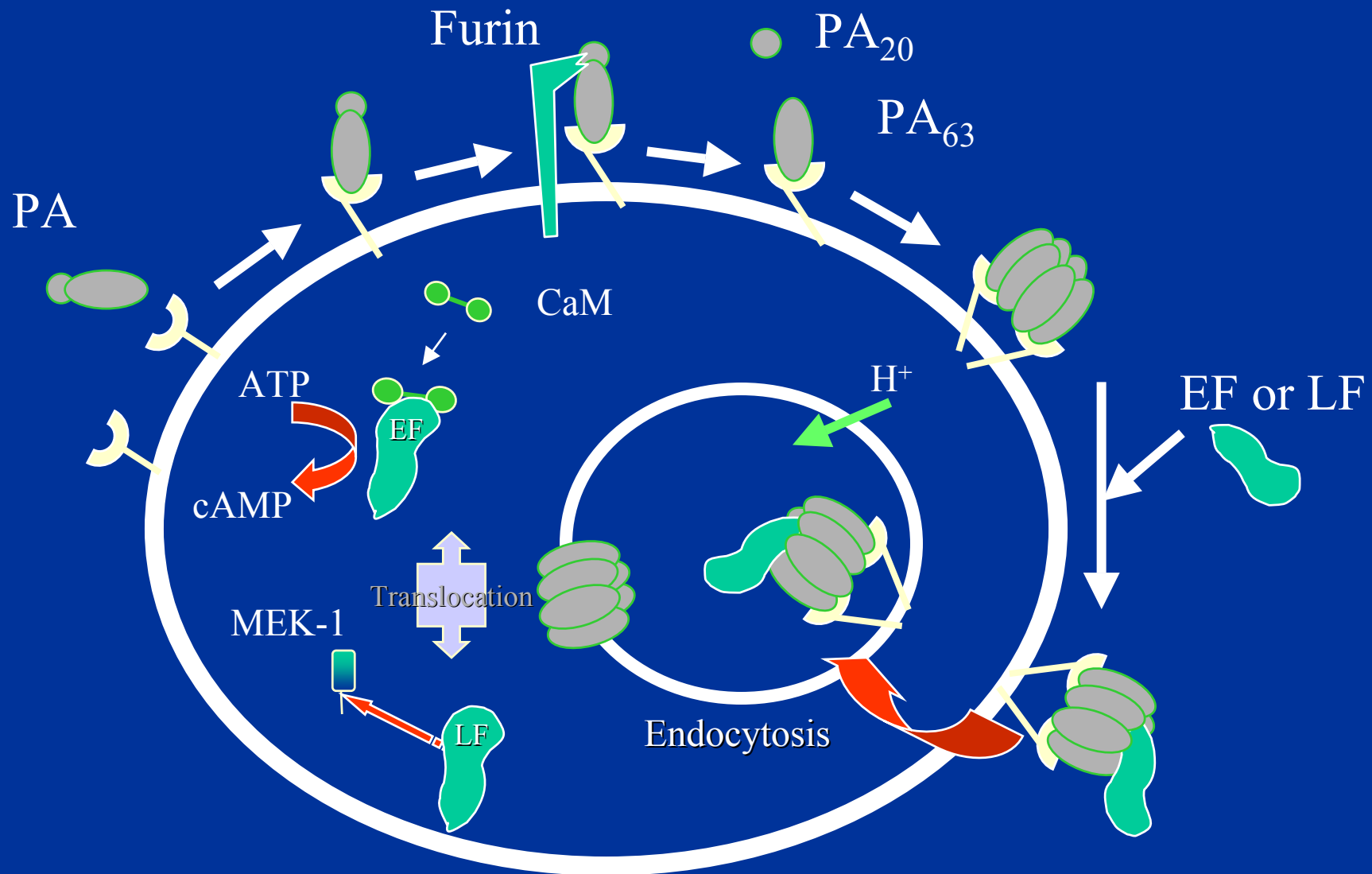
Centre well contains
hyperimmune anti-live
spore vaccine
antiserum

Anthrax Binary Toxins



Anthrax Toxin - Mode of Action

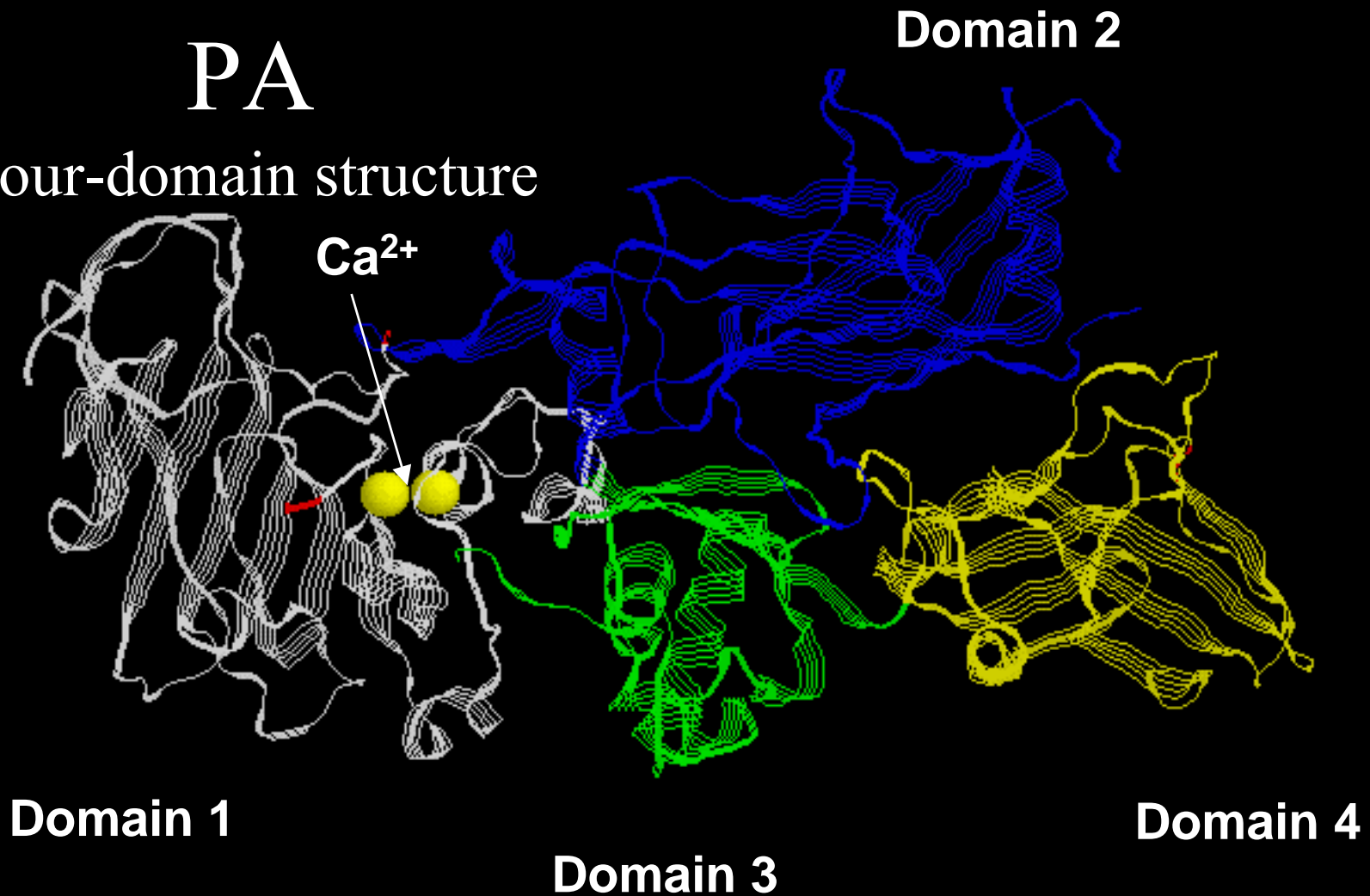
(from Leppla, 1999)



Anthrax Toxin

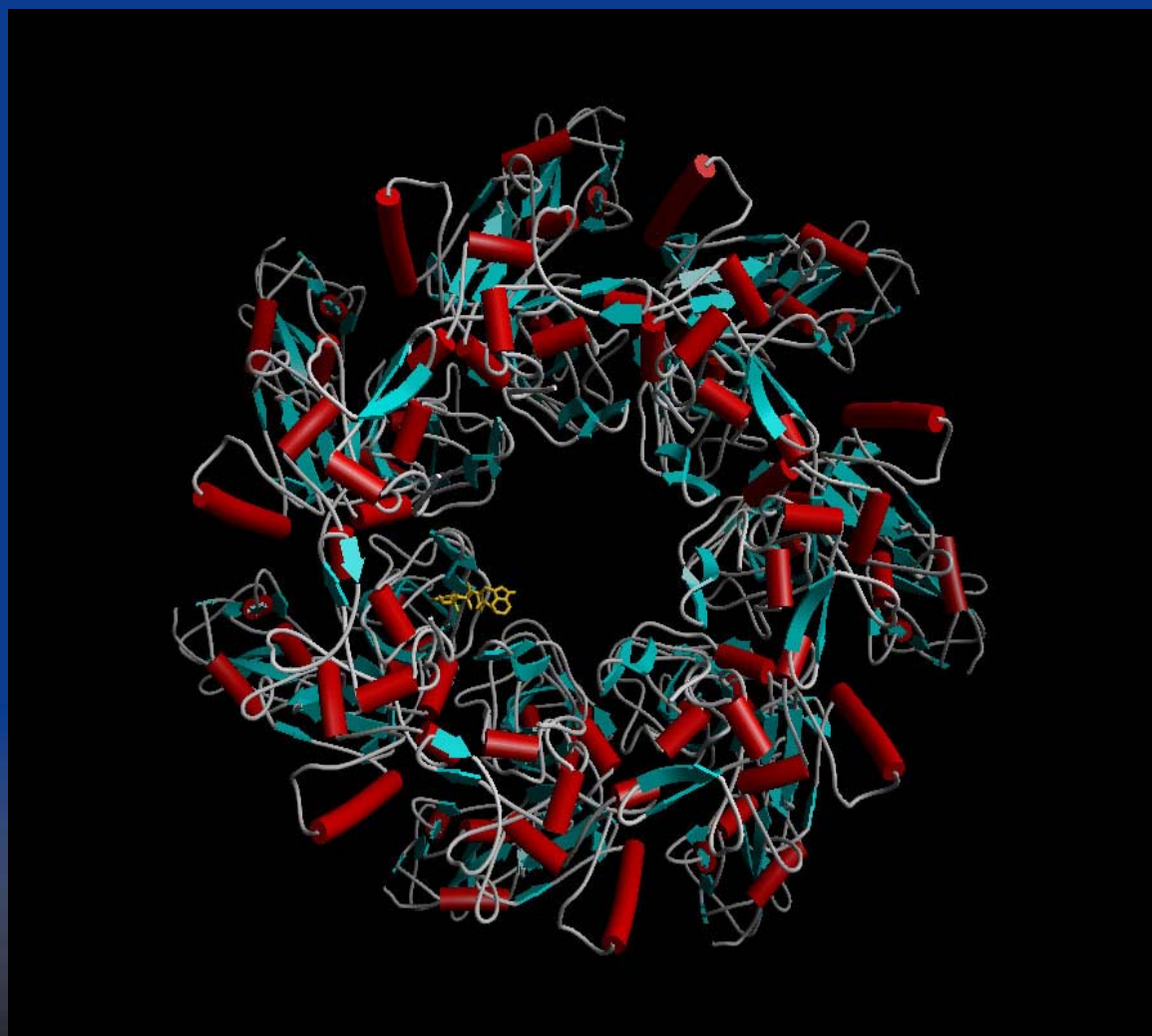
PA

Four-domain structure





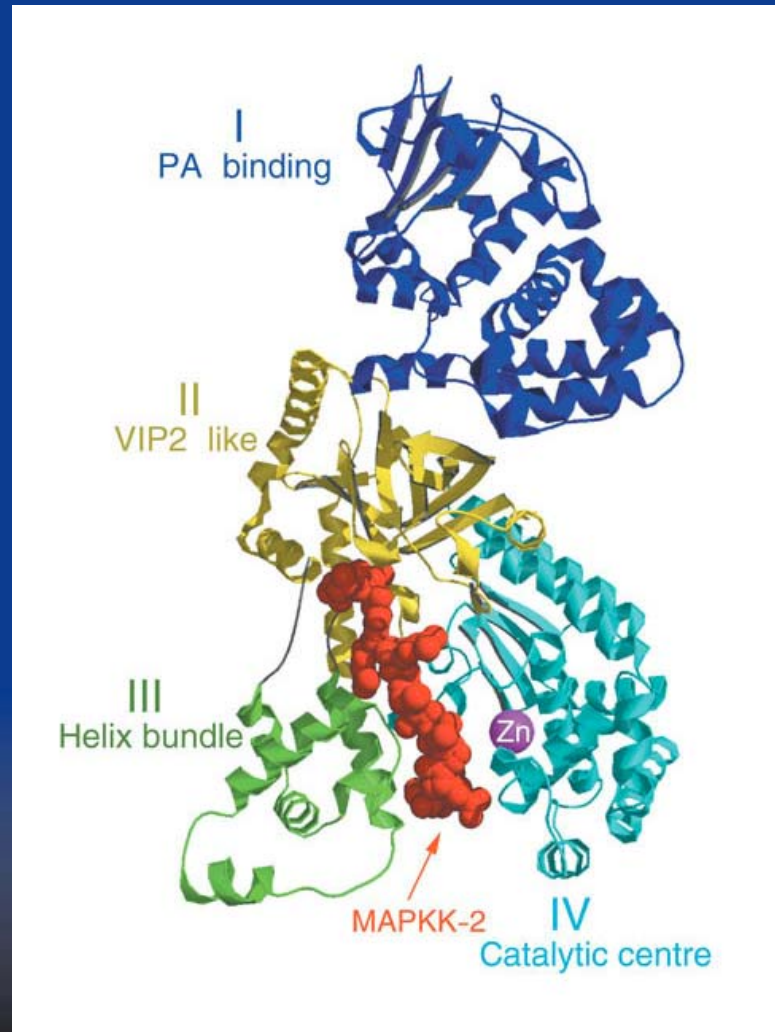
PA63 Heptamer



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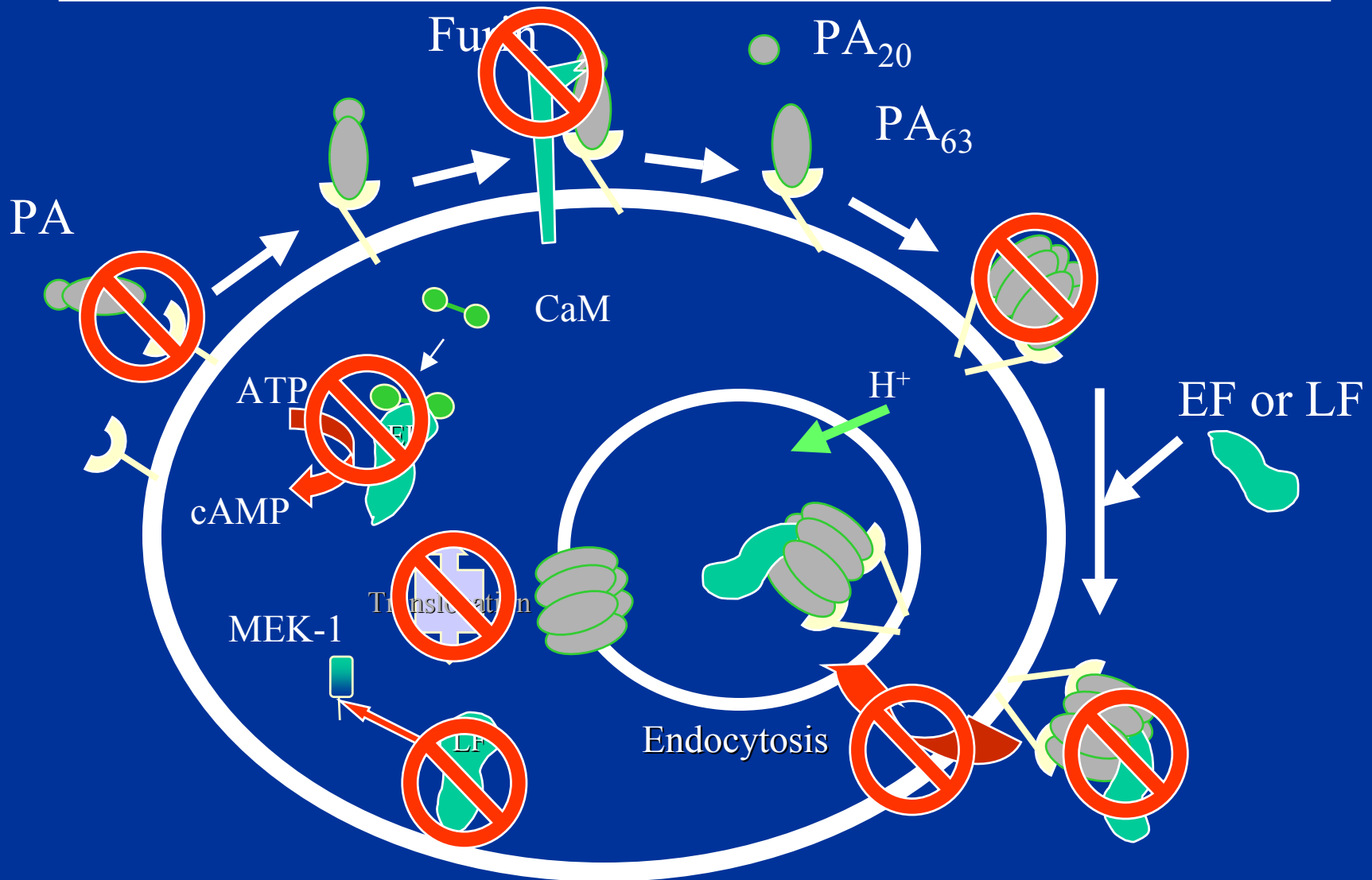
Lethal Factor (LF)



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Anthrax Toxin - Mode of Action

(from Leppla, 1999)



Small Inhibitor Molecules

- What's out there
 - Inhibitors of EF
 - Measurement of adenylyl cyclase activity
 - Quinazoline (Soelaiman et al., 2003)
 - Adefovir diphosphate (Shen et al., 2004)
 - Inhibitors of LF
 - Endoproteinase activity
 - Aromatic pharmacophores (Panchal et al., 2004)
 - Peptide inhibitors (Turk et al., 2004)
 - Green tea polyphenols/catechin (Dell'Aica et al., 2004)
 - Protein interaction inhibitors
 - Furin inhibitors
 - hexa-D-arginine amide (Sarac et al., 2004)
 - Complex inhibition
 - Polyvalent protein decoys (Mourez et al., 2002)
 - Dominant negative mutants (Sellman et al., 2001)

Immune Based Products

- Polyclonal anti-AVA
 - Little et al., 1997
- Monoclonal anti-AVA
 - Sawada-Hirai et al., 2004
- Monoclonal anti-PA
 - Wild et al., 2003
- Monoclonal anti-LF
 - Zhao et al., 2004

Immune Product Evaluation

- RFI announcement 12/24/03
 - Response by 2/1/04
 - Evaluation of candidate availabilities
 - Standardized technology platform
- *In vitro* Analyses
 - Anti-PA ELISA (human) binding assay
 - Lethal toxin neutralization (TNA)
 - Functional assay
 - Species independent



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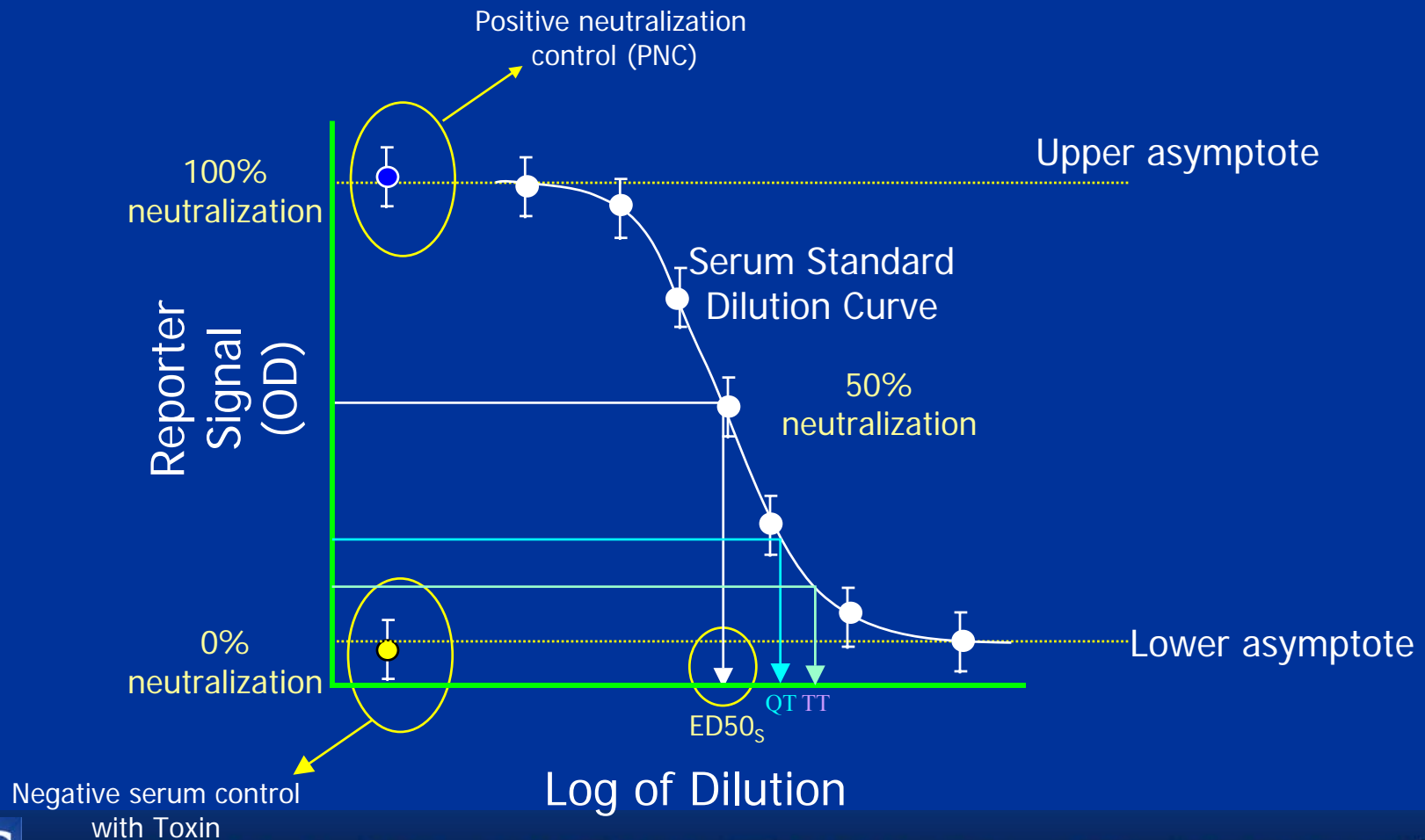
CDC Assay Features

- ELISA
 - Standardized technologies
 - Qualified reference standards & reagents
- TNA
 - Functional assay
 - Lethal toxin specific (PA + LF)
 - Not species or molecule dependent
 - Calculable endpoints
 - Mass value assignment by ELISA (AIG comparator)
 - Effective neutralizing concentrations
 - EC50
 - EC90, EC95, EC99
 - EC1, EC5, EC10
 - 4-PL curve parameters

TNA Endpoint Determination

- Assay Design -

50ng/ml PA; 40ng/ml LF; 3 x10E4 cells/well
Varied dilutions of Test material



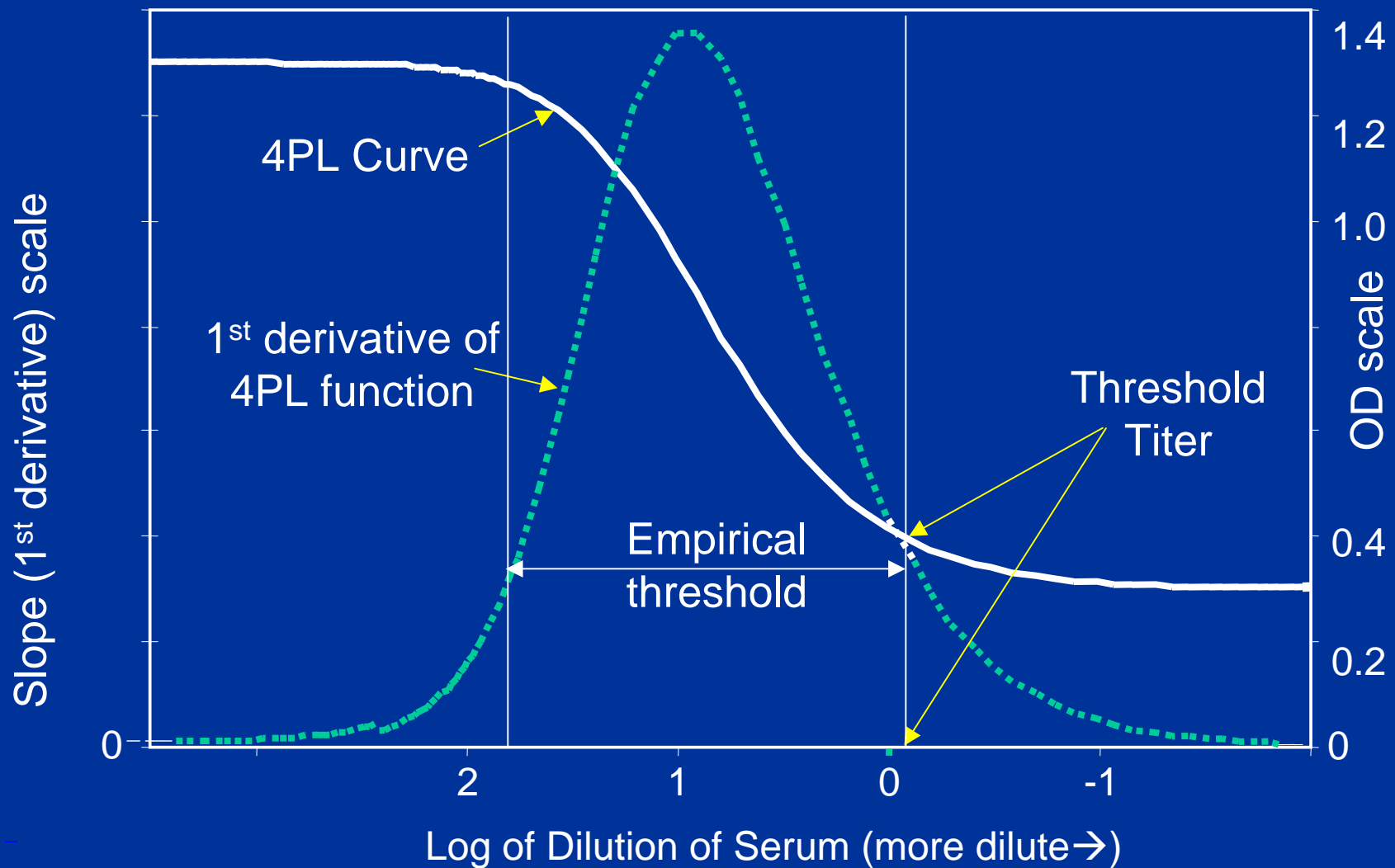
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1st & 2nd Derivatives

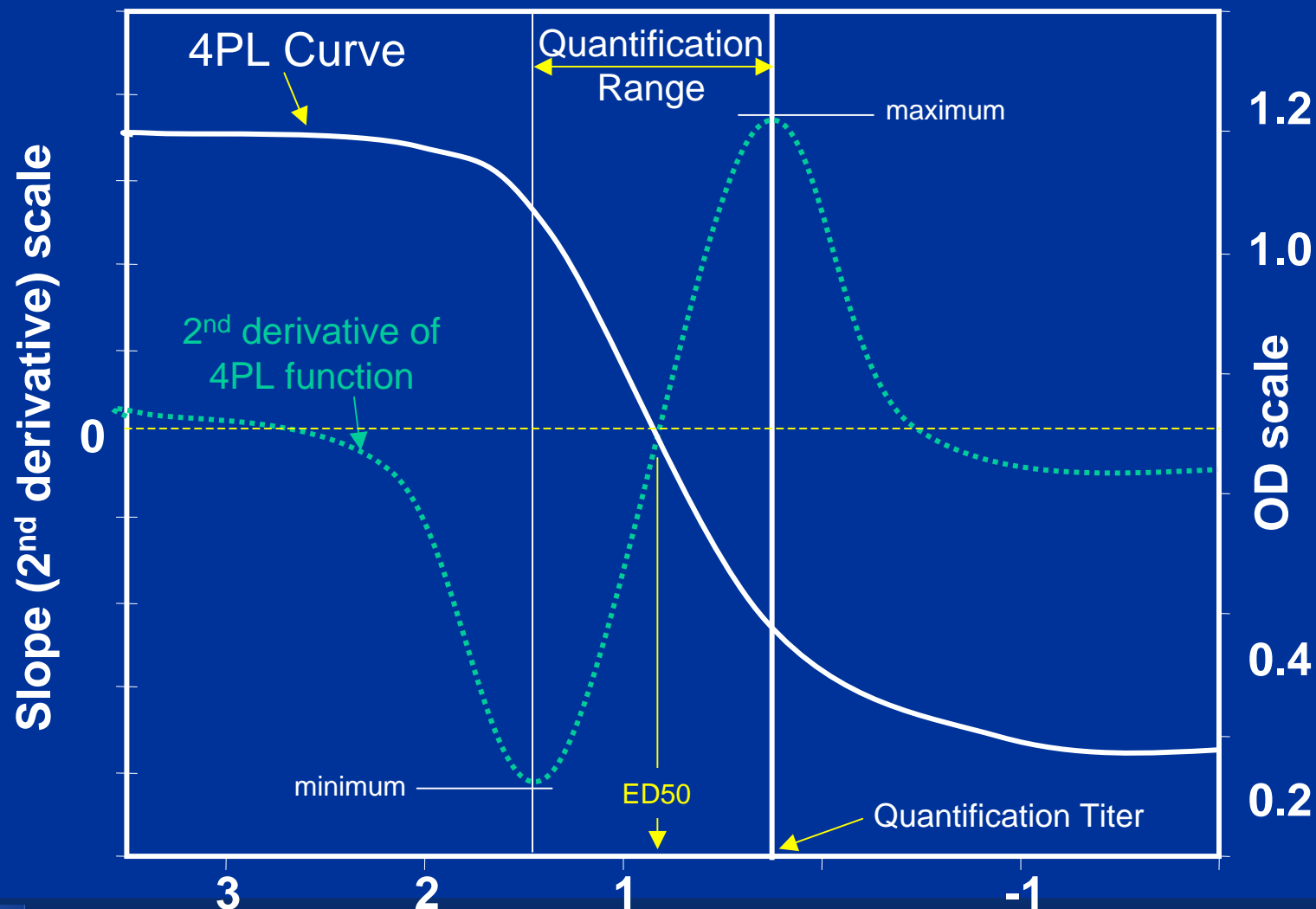
Define Reportable Values

- 1st Derivative
 - Measures the slope, and the changes in the slope, of the original function
- 2nd Derivative
 - Measures the rate of change of the slope in the original function (*i.e.*, acceleration)

Threshold Titer



Quantification Range & Quantification Titer

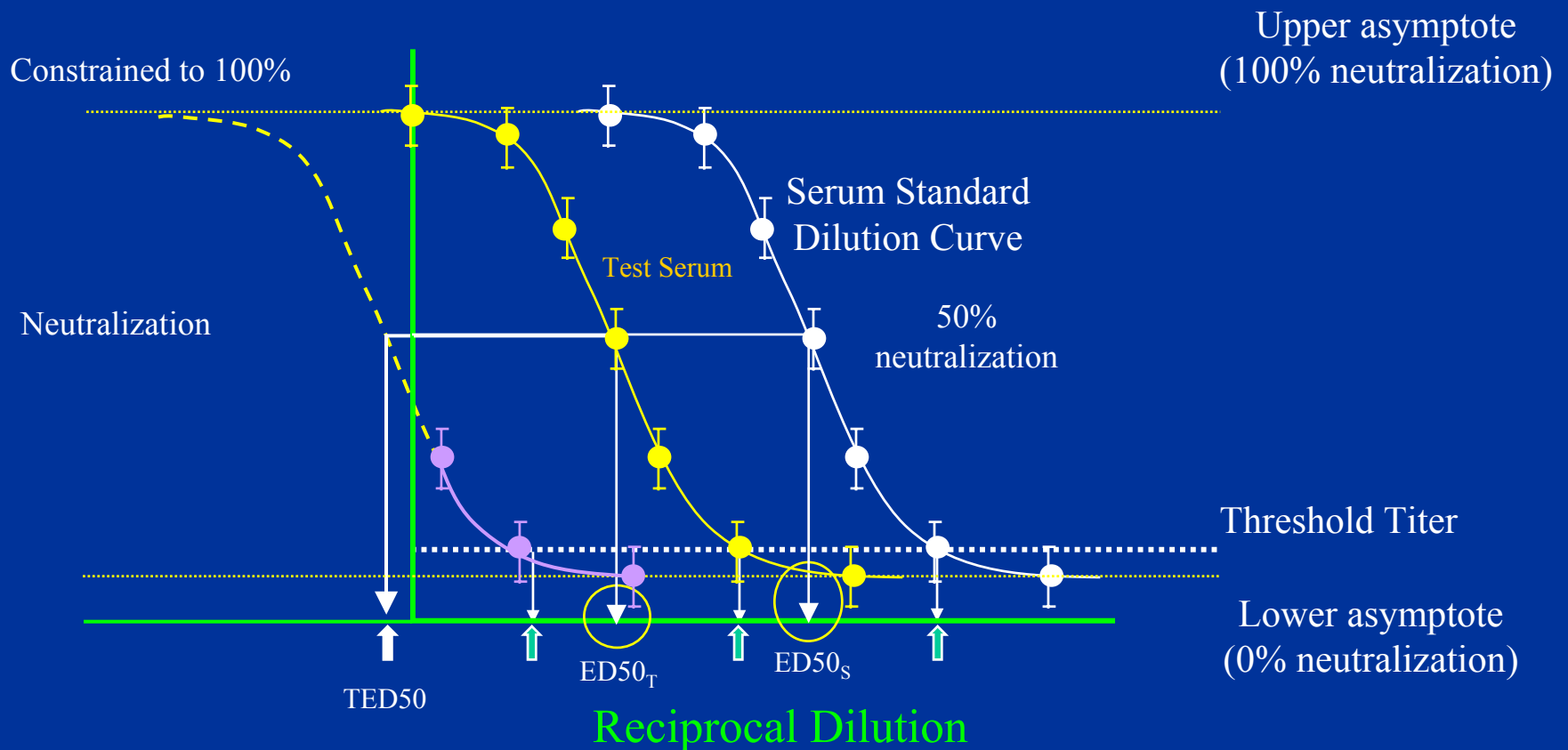


TNA Endpoints

- Effective Dilution 50% (ED50)
 - Defined by inflection point of 4-PL curve
 - Serum dilution where 50% of toxin is neutralized
- Threshold Titer (TT)
 - Highest serum dilution that gives a response significantly different than background
 - Determined by 1st derivative of 4-PL curve
- Quantification Titer (QT)
 - Highest serum dilution in the ‘usable’, ‘linear’ portion of the curve
 - More conservative estimate than the Threshold Titer
 - Defined by 2nd derivative of 4-PL curve

TNA Endpoint Determination

- Measuring Low Reactivity Sera -



Benefits of QT and TT

- Higher precision than other available methods
- Broad application to other assays including ELISA
- SAS-based program ('Taylor Method')
 - 4-PL model
 - High through-put analysis
 - QC criteria are consistently and objectively applied to all test plates

Rigorous QC Criteria

- Standard curve:
 - ED50 within expected range (mean \pm 2SD)
 - $R^2 > 0.950$
 - 3 dilution points on both sides of ED50 (full curve)
 - Sufficient depth of curve (> 0.6 OD)
 - Maximum OD of standard curve > 0.9 OD
 - Low variability in intra-dilution CVs ($< 20\%$)
- Negative serum control OD < 0.4 OD
- Positive Neutralization Control > 0.9 OD

TNA Performance Characteristics of Human Serum Standard AVR414 (n=96)

Intra-assay precision	7.7%
Inter-assay (intermediate) precision	23.6% CV=19.2% (Test sample 1) CV=27.9% (Test sample 2) CV=22.9% (Test sample 3) CV=24.2% (Reference std)
Goodness of Fit (R^2)	$R^2 = 0.992 \pm 0.005$
Mean ED50 _s	1161 \pm 281
Lower limit of detection	0.041 μ g/mL
Quantification Range	0.07 μ g/ml-0.30 μ g/mL

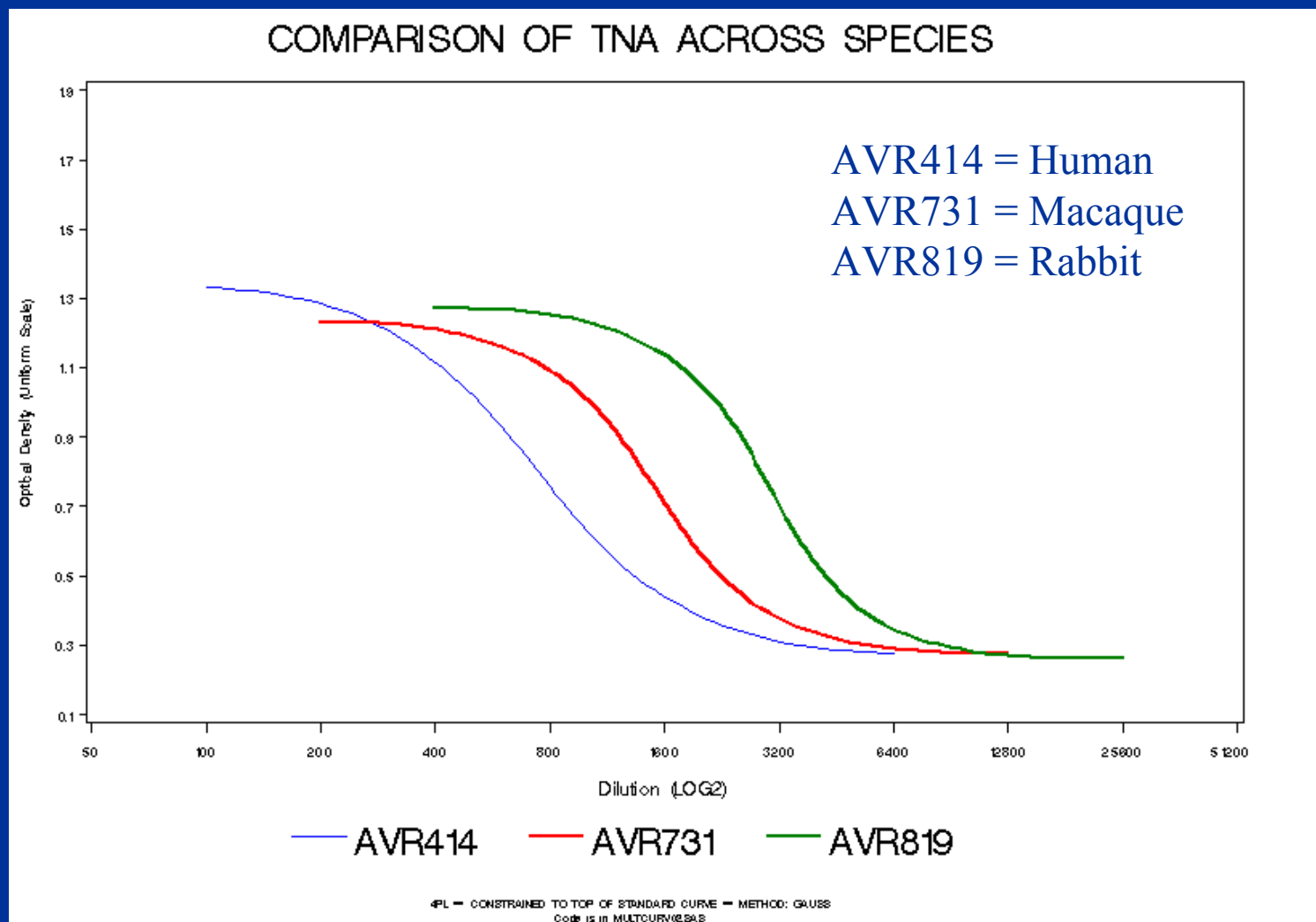


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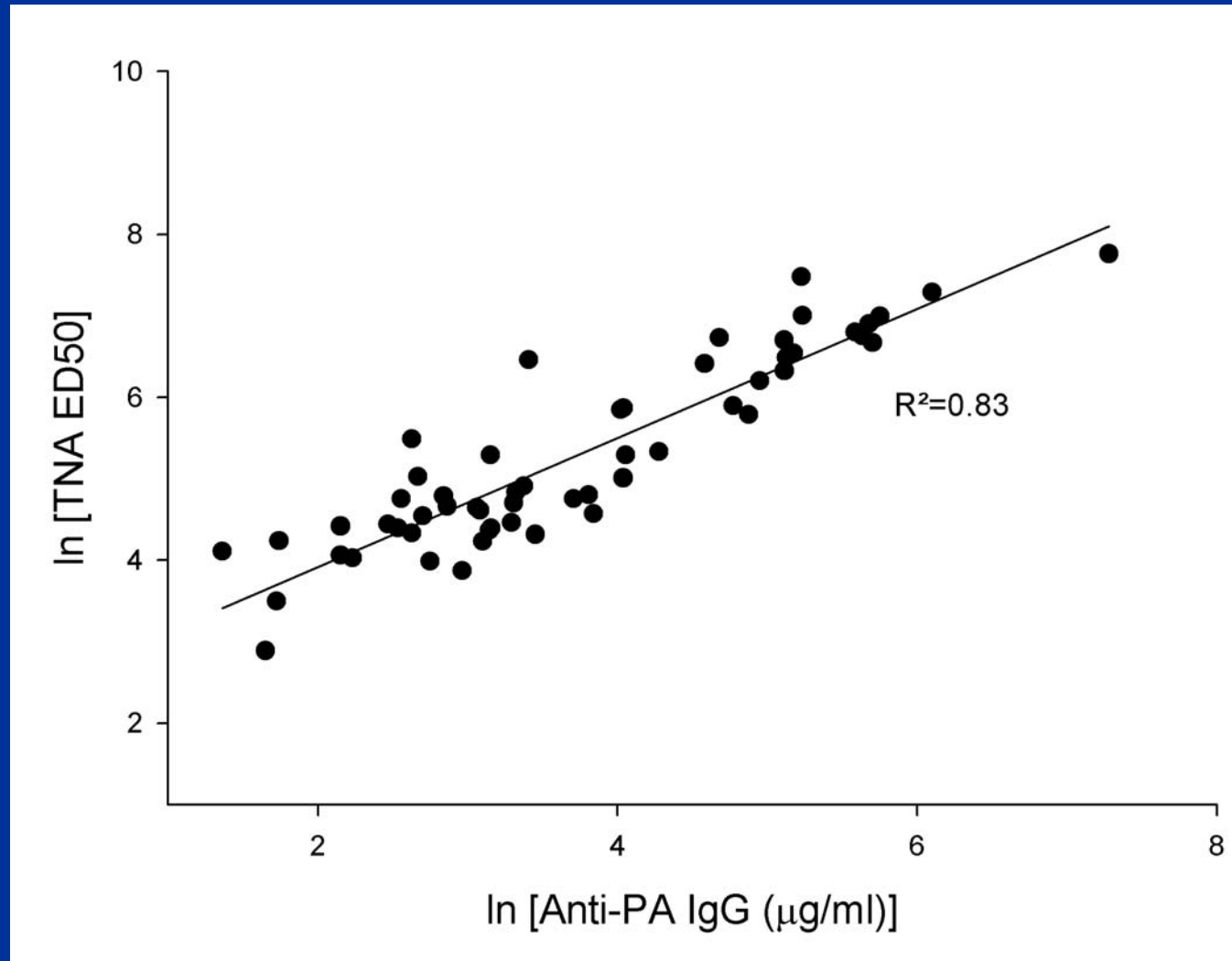
TNA is an Inter-Species Assay

- Human QC reagents
 - Using same standard reference and QCs as ELISA
 - Test various species
 - Macaque, mouse and rabbit
 - Anti-AVA, anti-rPA & MAbs
- Performance characteristics of 3 different animal species are similar

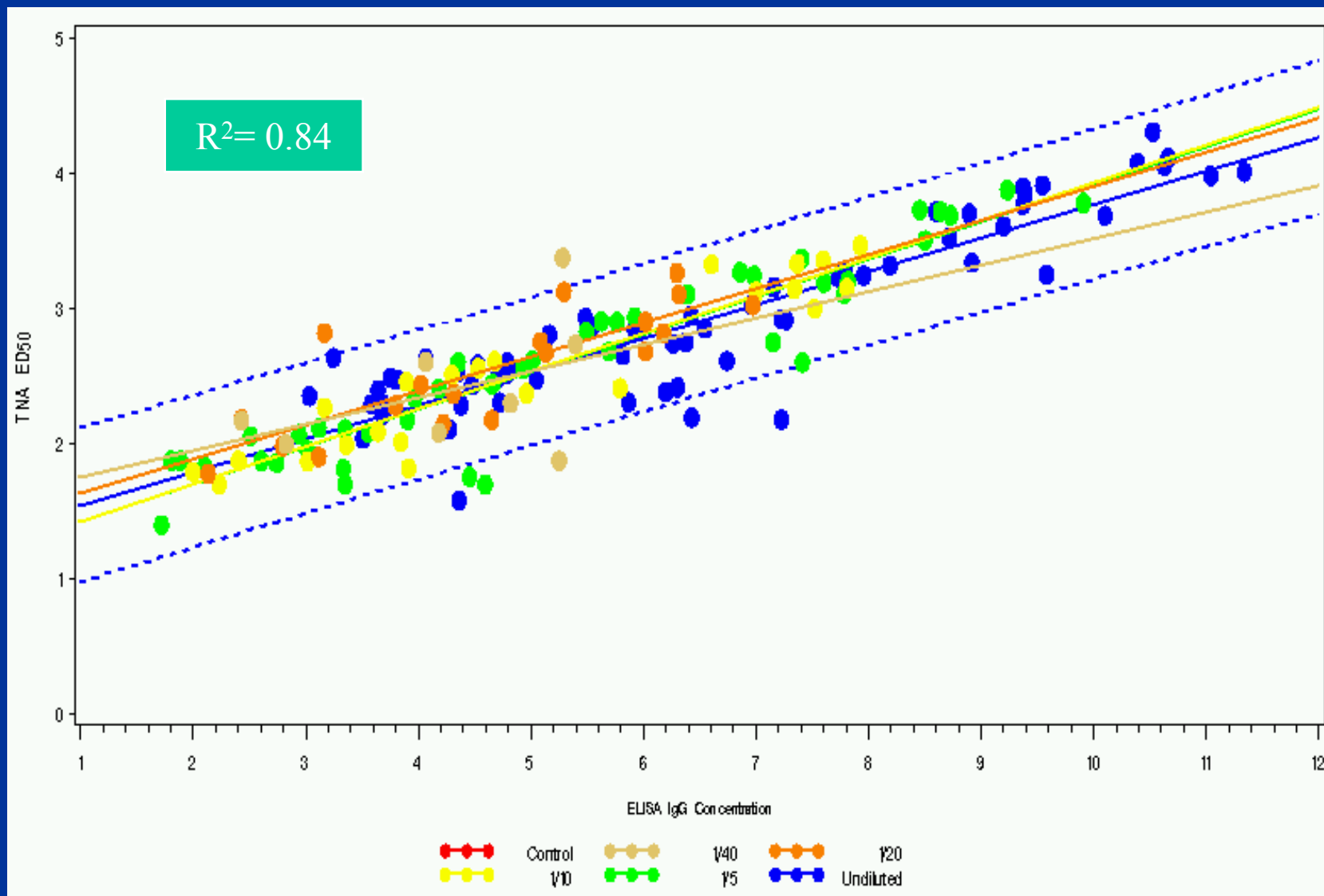
Human, Macaque, and Rabbit Standards



Correlation of Anti-PA IgG & Lethal Toxin Neutralization in Human Serum

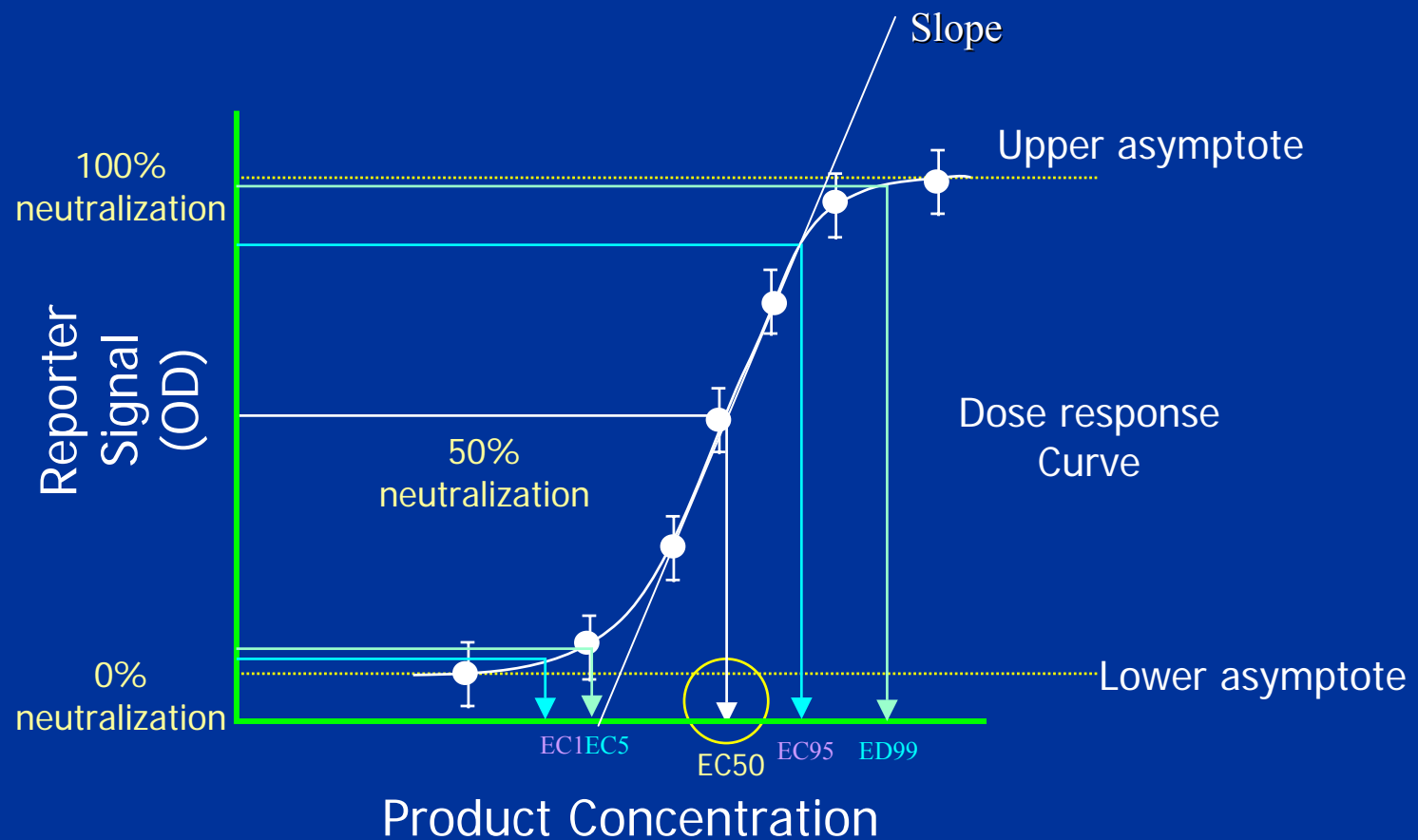


Correlation of Anti-PA IgG & Lethal Toxin Neutralization in Rhesus Macaque Serum



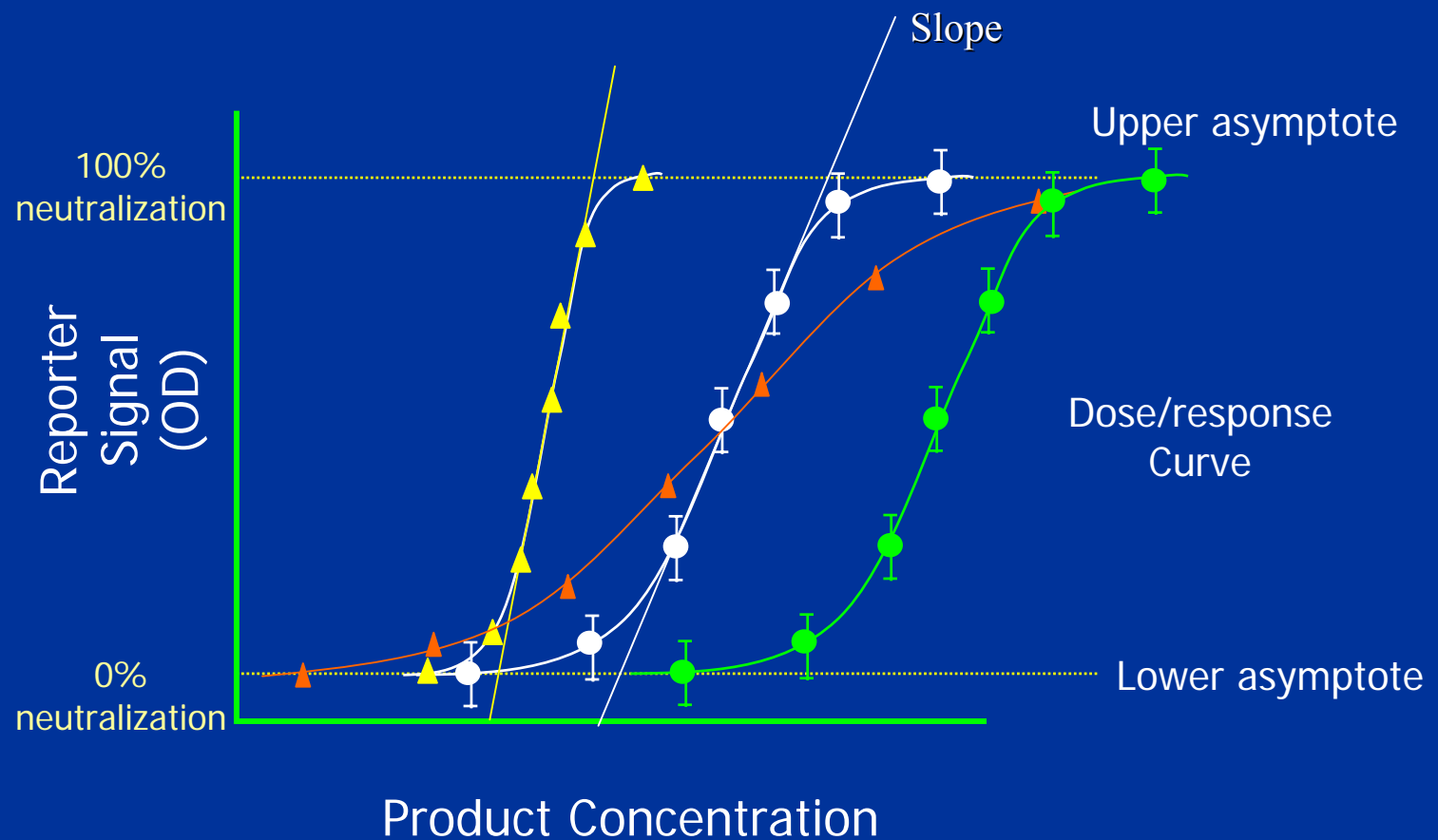
Immunotherapeutic Testing

- Reportable Values -

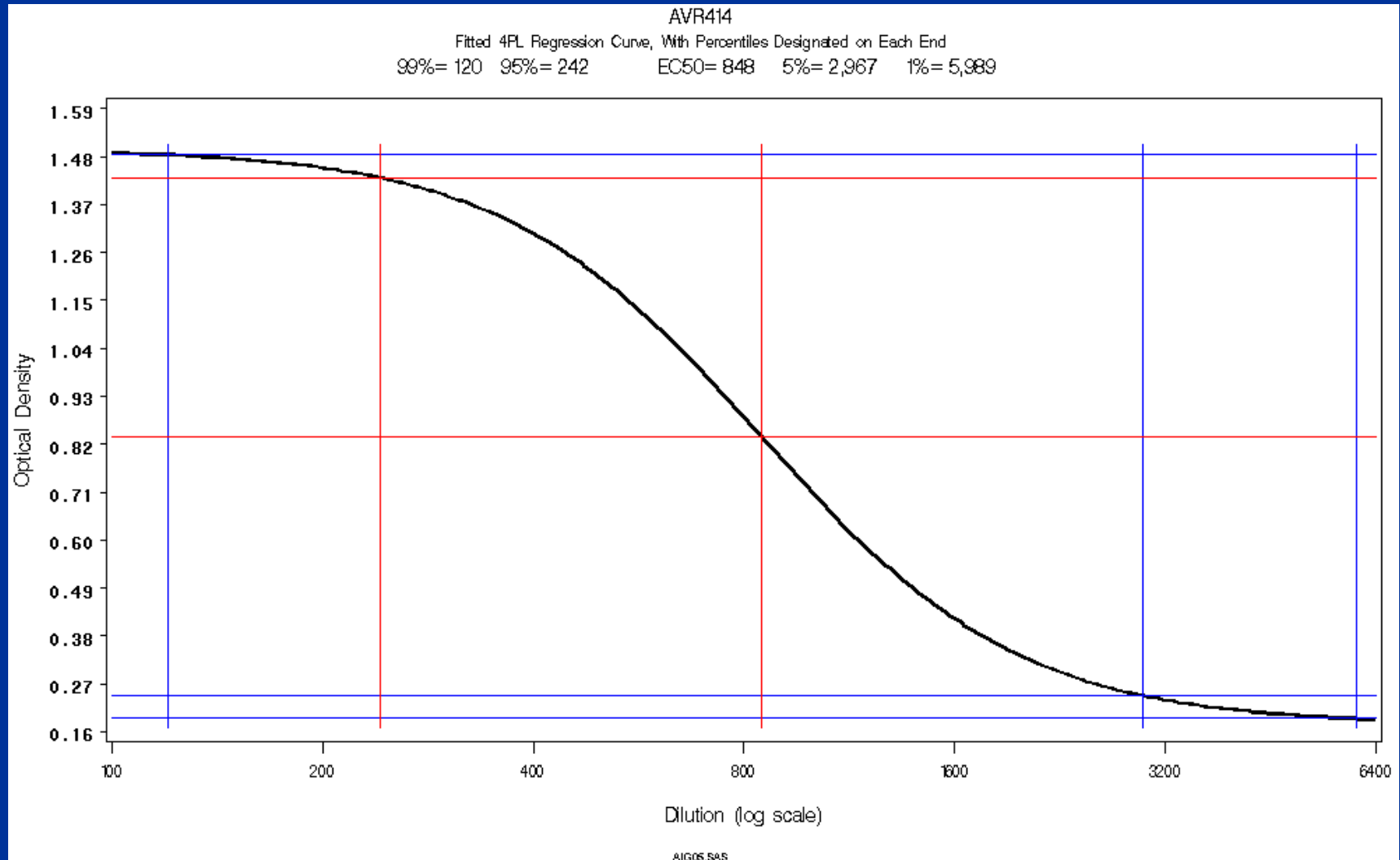


Immunotherapeutic Testing

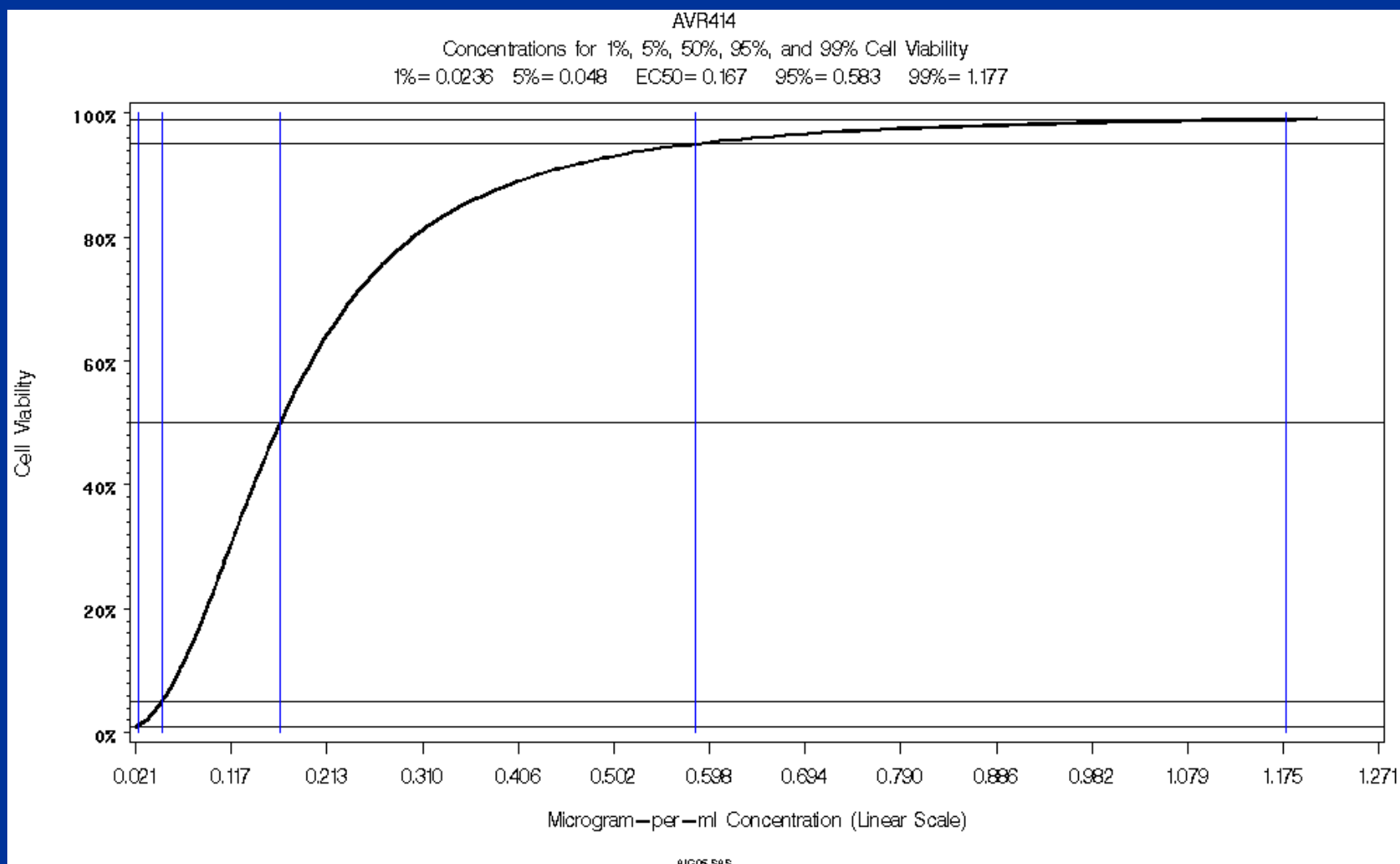
- *Comparative Curves* -



Human Reference Serum AVR414 (Transformed Data)



Human Reference Serum AVR414 (Untransformed Data)



Human Reference Serum AVR414

	Mean (n=6)	Std.Dev	CV
ED50 titer	818.82	65.86	8.04
Quantification Titer (1/dilution)	1496.25	176.11	11.77
Threshold Titer (1/dilution)	2525.00	437.50	17.33
99%	1.661	0.404	24.3%
95%	0.736	0.122	16.6%
EC50	0.176	0.015	8.7%
5%	0.043	0.008	17.9%
1%	0.020	0.005	24.9%
A - lower asymptote	0.235	0.046	19.6%
B - upper asymptote	1.482	0.200	13.5%
C - inflection point	12.442	1.088	8.7%
D - slope	2.097	0.232	11.1%



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Assay Limitations



- ELISA currently restricted to human antibody + Fc components
 - Excludes
 - Non-human antibodies
 - Fab, scFv, mimetics, small molecule inhibitors
- TNA
 - Assay emphasizes contribution of anti-PA 83kDa
 - Possibly limited to PA-, LF- or receptor-binding therapeutics
 - Modifications to broaden scope
 - LF emphasis
 - pre- VS. post-receptor events
 - Conformational dependence
 - Small molecule optimization

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Conclusion



- *In vitro* TNA assay
 - Accurate, precise, robust
- Standardized reagents & technologies
- Flexible application
 - Species independent
 - Quantifiable
- Limitations
 - PA emphasis
 - Optimized for antibodies
- Next steps
 - Relation to *in vivo* potency, product efficacy

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